

Research Article

***In Vitro* Plant Regeneration from Shoot Tip, Cotyledon and Nodal Explants of Biodiesel Plant (*Jatropha curcas* L.)**

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Abstract

In vitro regeneration of *Jatropha curcas* L. was established from different explants such as shoot tips, cotyledons and nodal segments using different concentrations and combinations of plant growth regulators and additives. The nodal segments were found superior explants to shoot tips and cotyledons. The highest result obtained on multiple shoot formation from nodal explants using 2.0 mg/l BA + 2.0 mg/l kin. in MS supplemented medium, in which 70% of explants responded for multiple shooting after 30 days of inoculation period. The average number of shoots and leaves/explants were 4.2 ± 0.64 and 2.90 ± 0.42 in this medium 90 days after inoculation respectively. The average shoot length of 3.62 ± 0.29 cm was also found in this same media and condition. Regenerated shoots were rooted well when transferred to of half strength of MS + 2.5 mg/l IBA, in which 50% of shoots responded after 30 days of inoculation period. The average number of roots/in-vitro raised shoots of 3.2 ± 0.65 and the average root length of 3.5 ± 0.29 cm were recorded in this medium after 60 days of inoculation. Plantlet regeneration was observed with low frequency in shoot tip and cotyledon explants. Most of the regenerants initially produced a clump of meristems and tended to form calli rather than multiple shoot formations.

Keywords

Jatropha, Shoot Tip, Cotyledon, Nodal Fraction, Regeneration

1. Introduction

Biodiesel (*Jatropha Curcus* L.) is a multipurpose, non-edible, oil yielding, perennial shrub or tree plant that belongs to the family Euphorbiaceae and grows well in the tropical and subtropical region in the world including Bangladesh. Besides, the plant can grow anywhere including soil considered infertile for food production, is adapted to harsh conditions and climate and also can live for about 50 years [9, 16, 21]. The most important aspect of this plant is its large

potential for bio fuel production, owing to high oil content of the seed, rapid growth and stiffness of the plant [25]. These phenomena have intensified interest in biodiesel as an environmentally sustainable alternative to fossil fuels because of rising global demand for renewable energy sources. Seed contains about 30-35% of nonedible oil which can be act an efficient substitute for diesel engines [5, 11, 13-15]. The oil derived from the seeds directly can be used as a replacement

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for kerosene cooking fuel, to use light lamps and for small family machineries in rural areas [7]. The kerosene oil can be converted into biodiesel for use in engines and 1000 grams of kerosene oil can produce 980 grams of pure biodiesel [20]. The most optimized practical process yield is around 94% of biodiesel [2] which emits 80% less CO₂ and 100% less SO₂ than fossil diesel [38]. Cultivation of *Jatropha* in one hectare of land can sequester around 20 tons of CO₂ annually [6]. *Jatropha* oil is an environmentally safe, cost-effective, renewable source of non-conventional energy and a promising substitute for diesel, kerosene and other fuel oils. Moreover, various parts of *Jatropha* such as latex, oil, twigs, wood, leaves are reported to use externally for healing wounds, to stop bleeding, to treat skin disease, rheumatism and other medicinal treatments [1, 8, 27, 28, 31, 36]. The plant *Jatropha* can be propagated from seeds, branch cuttings, grafting as well as by tissue culture. Thus, the use of this plant for large-scale biodiesel production is of great interest with regards to solving the energy shortage, reducing carbon emission and increasing the income of farmers in addition to its use in traditional medicines. To meet the large-scale demand and ensure easy supply of this elite material for sustainable cultivation, there is a need to establish clonal seed orchards and to develop mass multiplication techniques. The major constraints for plant propagation through stem cuttings are limited due to seasonal barrier as well as susceptibility to diseases [34]. Though seeds are due to low yield and poor germination and also the creation of genetic variation is a problem as the plant poses artificial and natural hybrids readily. Therefore, tissue culture technology can provide an alternative way to substantiate the rapid multiplication of elite clone and disease-free propagules for continuous supply of planting material with economic viability. Moreover, it will be used as a platform for genetic transformation and molecular breeding initiatives to improve stress tolerance and oil quality. Conservation of germplasm and the creation of somaclonal variation is also useful for *in vitro* culture system. Reports are available on *in vitro* propagation of *Jatropha Curcas* from different explants including node, shoot tip, cotyledon, hypocotyls, epicotyls, axillary bud proliferation and somatic embryogenesis [10, 19, 29-31, 35, 37]. Thus, the present study was undertaken to develop an efficient and reproducible *in vitro* plant regeneration protocol for *Jatropha Curcas* using different explants with different concentrations and combinations of plant growth regulators. This study will help to contribute the sustainable production of biodiesel and promote global efforts to develop cleaner and greener energy sources.

2. Materials and Methods

Shoot tip and cotyledon explants were inoculated from two weeks old *in vitro* derived plantlets and nodal explants were inoculated from 3 years old field growing plant at the experimental field of Plant Biotechnology and Genetic Engineering Division, IFRB, AERE, Savar, Dhaka. Nodal explants

were washed with distilled water, followed by washing with a few drops of detergent Trix and kept under running tap water for 30 minutes. Then the explants were soaked in 0.1% mercuric chloride (w/v) for 10 minutes and thoroughly washed four times with sterile distilled water in the laminar airflow cabinet. Surface sterilized nodal explants and *in vitro* derived shoot tip & cotyledon were inoculated on MS medium supplemented with different concentrations of 6-Benzylaminopurine (BA), Thidiazuron (TDZ) and Naphthalene acetic acid (NAA) alone and in combinations with BA + Kinetin (Kin), BA + Indole-3-butyric acid (IBA), BA + TDZ and BA with different additives of adenine hemisulphate (Ads), proline and glutamine for multiple shoot induction. Subcultures were performed at 30-day intervals to enhance numerous healthy shoots. Individual healthy shoots were excised and placed in ½ strength of MS media containing varying concentrations of IBA, Indole-3-acetic acid (IAA), and NAA for root initiation. The basic medium was used composed of MS (Murashige and Skoog, 1962) salts and organic compounds, 30g/l sucrose and 8 g/l agar. The pH was adjusted to 5.8 and then autoclaved for 20 minutes at 121 °C and 1.07 kg/cm². Cultures were incubated at 25 ± 2 °C with a 16 hours illumination of 50 µmol/cm²/s provided by cool white fluorescent tubes. The studies were performed with 10 explants for each medium type and replicated thrice. Data on various characters were collected on 90 days for multiple shoot proliferation and day 30 for rooting of *in vitro* produced shoots. *In-vitro* raised plants investigated every other day. The experiments were arranged in a completely randomized design (CRD). A descriptive analysis was carried out using the recorded data. Each value represents mean ± standard errors.

3. Results and Discussion

In the present investigation, different types of explants responded differently to the different media types (Table 1). Comparatively better response to shoot bud proliferation was observed on nodal explants using MS + 2 mg/l BA + 2 mg/l kin, in which 70% of explants induced multiple shoot inoculation after 30 days. The average number of shoots per explant was 4.2 ± 0.64, the average shoot length was 3.62 ± 0.29 cm and the average leaf number was 2.90 ± 0.42 in this medium inoculation after 90 days. The nodal explants initially produced a clump of meristems and gradually formed multiple shoots after 45 days of inoculation. Meanwhile, the shoot tip and cotyledon explants induced shoots with basal callus at the same inoculation period. The addition of BA with IBA has been reported to regenerate multiple shoots from nodal explants [35]. In this study, nodal explants were promoted to induce only callus by adding BA + IBA in the media. This might be due to the genetic makeup of the explants or an effect of genotypes. The nodal explants demonstrated the highest frequency of shoot proliferation, potentially due to the presence of a growth-promoting factor. The nodal explant was found superior to shoot tip and cotyledon explants for multi-

ple shooting and the combination of BA + Kin. was also investigated and reported to be more effective for multiple shoot induction of *Jatropha Curcas* L. [26]. A similar kind of result was also observed in this study. Supplements of Ads, proline and glutamine for regeneration into shoots of many crops have also been reported [26]. This data is in agreement with the present investigation. In our study, NAA alone or in combination with NAA + TDZ produced multiple shoots, which is close to the previous report of [23]. Using BA + IBA in the inoculation media for multiple shooting from shoot tip explants has been reported [20], which is also supported by the present investigation. It was observed in the present investigation that in combination with BA + kin., BA + IBA and

NAA + TDZ in MS-supplemented media, collagenesis of explant tissue was inhibited, and the difficulties of shoot elongation were also overcome, as explants were initially proliferated with a compact clump of meristems. It serves as evidence in the present investigation. [24] found superior shoot proliferation from cotyledonary petiole explants using TDZ on MS supplemented media. Differences in results reported by various authors with the various explants might be due to the concentrations of endogenous plant growth regulators and their metabolism occurring with the explant tissue. Difficulties of shoot elongation and profuse callusing at the basal end of proliferated shoots were also reported [22, 23]. A similar observation was made in the present investigation.

Table 1. Effects of different concentrations and combinations of auxins and cytokinins in addition with Ads for multiple shoot formation of *Jatropha Curcas* L. using different explants inoculated after 120 days.

Explants	Various concentrations and combinations of auxins & cytokinins with Ads and proline (mg/L)	Explant responded to form multiple shooting (%)	Average shoot/explant (no.) Mean $\pm$ SE	Average shoot length (cm) Mean $\pm$ SE	Average leaf (no.) Mean $\pm$ SE
Shoot tip	MS+2.5 BA	10	1.0 $\pm$ 0.01	1.5 $\pm$ 0.03	1.0 $\pm$ 0.01
	MS+5.0 BA	10	1.0 $\pm$ 0.01	1.5 $\pm$ 0.02	1.3 $\pm$ 0.01
	MS+1.0 BA+0.5 IBA	50	2.3 $\pm$ 0.32	2.6 $\pm$ 0.16	1.7 $\pm$ 0.52
	MS+2.0 BA+0.5 IBA	20	1.0 $\pm$ 0.01	1.3 $\pm$ 0.03	1.0 $\pm$ 0.01
	MS+2.0 BA+ 2.0 Kin.	10	1.0 $\pm$ 0.01	1.6 $\pm$ 0.02	1.0 $\pm$ 0.01
	MS+0.5 NAA	10	1.0 $\pm$ 0.01	1.4 $\pm$ 0.02	1.4 $\pm$ 0.01
Cotyledon	MS+1.0 NAA	40	2.4 $\pm$ 0.26	2.9 $\pm$ 0.29	1.6 $\pm$ 0.49
	MS+0.5 NAA+0.5 TDZ	60	2.9 $\pm$ 0.25	2.8 $\pm$ 0.39	2.1 $\pm$ 0.28
	MS+1.0 NAA+0.5 TDZ	10	1.0 $\pm$ 0.01	1.2 $\pm$ 0.01	1.3 $\pm$ 0.02
	MS+2.0 BA+ 2.0 Kin.	None	None	None	None
Nodal fraction	MS+1.0 BA+20 Ads.	10	1.0 $\pm$ 0.01	1.6 $\pm$ 0.03	1.2 $\pm$ 0.01
	MS+2.0 BA+20 Ads.	10	1.0 $\pm$ 0.01	1.2 $\pm$ 0.02	1.3 $\pm$ 0.01
	MS+2.0 BA+0.5 IBA	10	1.0 $\pm$ 0.01	1.3 $\pm$ 0.02	1.2 $\pm$ 0.01
	MS+1.0 BA+2.0 Kin.	30	2.6 $\pm$ 0.52	2.5 $\pm$ 0.46	1.5 $\pm$ 0.02
	MS+2.0 BA+ 2.0 Kin.	70	4.2 $\pm$ 0.62	4.6 $\pm$ 0.23	3.1 $\pm$ 0.42

The rooting response varied depending on the concentrations and combinations of various auxins utilized (Table 2). Well-developed shoots ranging in length from 4.0 to 4.5 cm were excised individually and transferred to half strength of MS media supplemented with different concentrations and combinations of IBA, IAA and NAA for root initiation. Among the auxins used, IBA was shown to be the most effective for root induction and 2.5 mg/l IBA was found to be the most ideal, whereas half of the shoots rooted within 30

days of the inoculation period. In the same media of MS + 2.5 mg/l IBA, the average number of roots/shoot was induced 3.2 ± 0.65 and the average root length was 3.5 ± 0.29 cm. Related findings were reported for *Jatropha in vitro* rooting [20, 24, 26]. The superiority of IBA for rooting over other auxins has been reported [3, 4, 12, 17]. The effectiveness of IBA on the rooting of *in vitro* raised shoots of other crops has been reported with the earlier finding [18, 32, 33]. [26] obtained superior root induction in *Jatropha Curcas* using a combina-

tion of IBA+NAA. This is also consistent with our findings in this investigation.

Table 2. Effects of different concentrations and combinations of IBA, IAA and NAA on ½ strength of MS supplemented media for root initiation from *in vitro* proliferated shoots of *Jatropha Curcas* L. inoculated after 30 days.

Auxins (mg/L)			Shoot responded to root induction%	Root/shoot	Root length
IBA	IAA	NAA		(No.) Mean ± SE	(cm) Mean ± SE
0.5	---	---	---	---	---
1.0	---	---	---	---	---
1.5	---	---	---	---	---
2.0	---	---	30	2.1±0.36	2.5±0.46
2.5	---	---	50	3.2±0.65	3.5±0.29
---	1.5	---	---	---	---
---	1.0	---	---	---	---
---	1.5	---	---	---	---
---	2.0	---	---	---	---
---	2.5	---	---	---	---
---	---	0.5	---	---	---
---	---	1.0	---	---	---
---	---	1.5	---	---	---
---	---	2.0	---	---	---
---	---	2.5	---	---	---
0.5	0.5	0.5	---	---	---
0.5	---	0.5	20	1.2 ± 0.24	1.9±0.16
1.0	---	0.5	40	2.5 ± 0.45	3.4±0.42
0.5	0.5	---	---	---	---
1.0	0.5	---	---	---	---
1.0	0.5	0.5	---	---	---

Differential effects of root induction documented by different authors employing various concentrations and combinations of auxins which could be attributed to genotypes and diverse sources of explant utilized.

Rooted plantlets of 5.0 to 5.5 cm long were removed from the culture vessels and washed gently under running tap water to get rid of agar. polybags and in the field. In fact, the present investigation was made to develop an efficient *in vitro* re-

generation protocol of *Jatropha Curcas* L. from shoot tip, cotyledon and nodal explants using different concentrations and combinations of auxins and cytokinins and also various additives that could be beneficial for commercial cultivation of this plant without seasonal barriers and also for genetic enhancement through *in vitro* mutagenesis and genetic transformation with the gene of interest of this economically important biodiesel plant as well.



Figure 1. *In vitro* shoot bud induction and plant regeneration from shoot tips, cotyledon and nodal explants of *Jatropha curcas* L. a-c). Shoot bud initiation and multiple shoot proliferation from shoot tips after 1, 2 and 3 months of culture respectively. d-f). Multiple shoot formation from cotyledon after 2 and 3 months of culture respectively. g-h). Shoot bud initiation and multiple shoot proliferation from nodal segments after 1 and 3 months of culture respectively. i). Healthy shoot formation and shoot elongation on transferring to initiated shoots after 2 months of culture. j). Root induction on half strength MS + 2.5 mg/l IBA after 1 month of culture. k). *In vitro* raised plant resumed new growth in the polybag.

The *in vitro* raised plantlets showed 90% survival during hardening and acclimatization period of 30 days. The transplanted plantlets established well in field condition.

KIN
TDZ
ads

Kinetin
Thidiazuron
Adenine Hemisulphate

Abbreviations

BA	6-Benzylaminopurine
NAA	Naphthalene Acetic Acid
IAA	Indole-3-acetic Acid
IBA	Indole-3-butyric Acid

Author Contributions

Md. Humayun Kabir: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing

Md. Monirul Islam: Data curation, Formal Analysis, Investigation, Writing – review & editing

Pronabananda Das: Data curation

Mazedul Anwar Razib: Data curation, Formal Analysis, Investigation, Methodology

Ahmad Nazri Karim Mamun: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing

Conflicts of Interest

The authors declare no conflicts of interest.

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